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Regulation of protein synthesis in Tetrahymena: isolation and characterization of polysomes by gel filtration and precipitation at pH 5.3

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ABSTRACT

The fraction of ribosomes loaded on polysomes is about 95% in logarithmically growing Tetrahymena thermophila, and about 4% in starved cells. Cytoplasmic extracts from cells in these two physiological states were used to develop column chromatographic methods for the purification of polysomes. Bio-Gel A 1.5 m was found to separate total cytoplasmic ribosomes from many soluble proteins, including RNAse, with no detectable change in the polysome size distribution. Polysomes can be separated from monosomes and non-polysomal mRNA by chromatography on Bio-Gel A 15 m without size selection. These methods can easily be adapted to large scale preparations of polysomes, even from cells where a small fraction of the ribosomes is on polysomes. A method is described for reversible precipitation of polysomes and monosomes from dilute solutions at pH 5.3 which greatly facilitates polysome isolation. Hybridization of ^{3}H-labeled polyU to RNA isolated from column fractions has been used to demonstrate that purification of EDTA released polysomal mRNA can be performed using the column chromatography procedures described here. These methods have been employed to demonstrate that most of the cytoplasmic mRNA in log-phase Tetrahymena is loaded onto polyribosomes while most of the mRNA in starved cells exists in a non-polysomal form.

INTRODUCTION

Many studies on the regulation of gene expression would be facilitated by simple methods for isolating large amounts of polysomal and non-polysomal mRNA. For example, characterization of specific gene expression by nucleic acid hybridization frequently requires purified polysomal mRNA (for review, see ref. 1). Efficient separation of polysomal and non-polysomal RNAs is particularly important in the analysis of translational control of gene expression, especially where only a small fraction of the mRNA is loaded on polysomes. Isolation of polysomes is also required for immunoprecipitation of polysomes translating specific mRNAs since polysomes must be free of soluble cell proteins which would compete with nascent polypeptides for antibody binding. Finally, the easy isolation of large amounts of polysomal and non-polysomal mRNAs would facilitate their characterization.

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Previous methods have largely utilized sedimentation through sucrose gradients for the purification of polysomal and non-polysomal mRNAs. Such methods have several limitations. They are generally time consuming, work intensive, and are difficult to apply to large scale preparation of polysomes from cells in which only a small fraction of ribosomes is in polysomes. The polysome fractions isolated are usually enriched in larger polysomes.

Recently, a gel-filtration method using Sepharose-2B was introduced to purify polysomes from Escherichia coli (2) and was extended to the purification of polysomes from Xenopus laevis (3). However, this method selects for larger polysomes, so that any mRNA found on small polysomes would be underrepresented.

We present here an improved method for purifying polysomal and non-polysomal RNA using gel filtration on Bio-Gel. This procedure is not selective for large polysomes. In addition we describe a method for reversible precipitation of polysomes at low pH which greatly facilitates their concentration from dilute solutions. We have used these methods to demonstrate differences in the loading on polysomes of cytoplasmic poly A (presumably reflecting the distribution of polyA+ mRNA) in exponentially growing and starved Tetrahymena thermophila.

MATERIALS AND METHODS

Cell growth and isotopic labeling

Tetrahymena thermophila (formerly T. pyriformis, syngen 1) were grown in medium containing 1% w/v proteose peptone (Difco), 0.2% dextrose (Fisher), and 0.003% sequestrene (Geigy) at 28°C to a density of approximately 2 x 10^5 cells/ml. Cells were starved in 10 mM Tris-HCl, pH 7.4, at 28°C for 12-18 hours at a concentration of approximately 1 x 10^5 cells/ml. All cultures were contained in Erlenmeyer flasks (0.5 to 4.0 l) filled to 20-30% of capacity and aerated by rotary shaking at 100-130 rpm. To label nascent polypeptides, starved cells were pulsed with 10 μCi each ^{3}H-leucine (63 Ci/m mole; NEN)/ml and ^{3}H-lysine (40 Ci/m mole; NEN)/ml for 1 minute just before harvesting. For some experiments, growing cells were continuously labeled with 2 μCi ^{3}H-uridine (40 Ci/m mole; NEN)/ml and 2 μCi ^{14}C-leucine (60 Ci/m mole; NEN)/ml. For subsequent starvation, cells were washed three times with 10 mM Tris-HCl, pH 7.4, and starved in the absence of added isotope.

Preparation of polysomes

For large-scale isolations 0.1 μg cycloheximide (Sigma)/ml was added to the cultures 3 to 5 min before havesting to inhibit run-off of ribosomes. For

small-scale analytical studies, cycloheximide addition is not desirable since it increases the average polysome size as much as 50% (F. Calzone, unpublished observation). Cells were collected at 1500 g in 50 ml conical tubes and washed 1-2 times with 10-50 times the pellet volume of fresh culture medium containing 0.1 μg cycloheximide/ml. The final cell pellet was gently loosened in a minimum volume of fresh culture medium and cells were lysed on ice by addition of 8-10 volumes of 30 mM Tris, 20 mM KAc, 50 mM $MgCl_2$, 2% spermidine tri-HCl (Sigma), 1 mg heparin-sulfate (Sigma)/ml, 50 mM aurin tricarboxilic acid (Sigma), 2 mM dithiothreitol (DTT), 10 μg cycloheximide/ml, 0.5% v/v NP-40 (BRL) adjusted to pH 7.1 with HAc. The concentration of ribosomes in this crude lysate was usually below 30-40 A_{260}. The lysate was kept on ice for 5-10 min, vortexed vigorously, and centrifuged at 10,000 g for 10 min. The resulting supernatant can be used immediately or stored indefinitely in 10-40% glycerol at -70°C.

Chromatography of polysomes

Bio-Gel A 1.5 m and A 15 m (100-200 mesh) were purchased from Bio-Rad. Analytical columns (1 cm x 54 cm) or preparative columns (4 cm x 70 cm) were developed at flow rates of 0.2-0.3 ml/min at 2.5°C in 25 mM Tris, 10 mM KAc, 25 mM $MgCl_2$, 0.2% spermidine tri-HCl, 10 μg cycloheximide/ml, 2 mM DTT, 10% v/v glycerol, 0.1% sodium azide, 0.2% antibiotic-antimycotic mixture (Gibco), adjusted to pH 7.1 with HAc. For some applications (e.g. immunoprecipitation) a simpler buffer (10 mM Tris, 10 mM KAc, 25 mM $MgCl_2$, 0.1% spermidine tri-HCl, 1 μg cycloheximide/ml, pH 7.1) can be used. Elution profiles were monitored at 254 nm with an LKB 2089 Uvicord III spectrophotometer. When cells were labeled with ^3H-uridine and ^{14}C-leucine, 1.5 ml fractions were collected, added to 8 ml Liquiscint (National Diagnostics), and the dpm for each isotope determined in a Beckman Model 7500 scintillation counter.

Precipitation of polysomes and monosomes at pH 5.3

To concentrate polysomes or monosomes, the pH of the appropriate fractions in column buffer was lowered to 5.3 by addition of 0.005 volumes of 2M KAc adjusted to pH 5.3 with acetic acid. After incubation on ice, usually for 15 min, the white precipitate was collected by centrifugation for 10 min at 10,000 g. The supernatant was removed and the pellet was resuspended in 50 mM Tris, 20 mM KAc, 50 mM $MgCl_2$, 0.1% spermidine tri-HCl, 1 μg cycloheximide/ml, 2 mM DTT, adjusted to pH 7.4 with HAc, during 1-2 hours on ice. After resuspension, undissolved material was removed by centrifugation for 5 min at 10,000 g. These supernatants may be stored in 40% glycerol at -20 to -70°C.

To test the effects of various parameters on precipitation of polysomes and monosomes, reactions were carried out in 1.5 ml Eppendorf tubes in 0.5 ml of 25 mM Tris, 10 mM KAc, 0.1% spermidine tri-HCl, 2 mM DTT, 1 μg cycloheximide/ml, 10% glycerol, pH 7.1, and varying concentrations of $MgCl_2$ (see Results). The efficiency of precipitation was calculated as A_{260} units in the pellet/total A_{260} units. Polysomes from Tetrahymena are contaminated with a glycogen-like material which has significant absorbance at 260 nm. This material co-isolates with RNA, but can be removed by centrifugation through CsCl (1.7 g/cc) at 38K rpm in a Beckman 50 Ti rotor for 48 hr at 23°C. The contaminant bands while the RNA pellets. Alternatively, the contaminant can be removed by centrifugation of RNA preparations at 100,000 g for 15 min In column buffer this material is found to have an A_{260}/A_{320} of approximately 2.5. All A_{260} measurements of ribosomes were corrected for contaminating material as follows: A_{260} ribosomes = A_{260} -2.5 A_{320}.

Chromatography of EDTA released polysomes

Precipitated column-purified polysomes were dissociated in 50 mM Tris-HAc, 0.2 M KCl, 10 mM EDTA, pH 7.4; 0.1 volume of glycerol was added and they were rechromatographed in this buffer.

Analysis of polysomes

Polysome profiles were obtained by sedimentation through 20-50% w/v linear sucrose gradients containing 25 mM Tris-HAc, 10 mM KAc, 25 mM $MgCl_2$, 1 μg cycloheximide/ml, 2 mM DTT, pH 7.4, in a Beckman SW 50.1 rotor at 40K rpm for 1 hr at 4°C. Gradients were then scanned at 254 nm using an Isco model UA-5 spectrophotometer with continuous-flow cuvette. When radioactivity was present, and column-purified polysomes were being analyzed, 0.25 ml fractions were collected and 5 ml of Liquiscient and 0.25 ml of water were added for scintillation counting. If polysomes were not separated from unincorporated isotope by prior chromatography on Bio-Gel, the radioactivity in each fraction was determined by TCA precipitation. The A_{254} tracings of polysome gradients were digitized and plotted with a Tektronix Model 4956 Digitizer and Controller.

The fraction of ribosomes loaded on polysomes and the mass average polysome size were determined from the polysome profiles after subtraction of EDTA-resistant background determined from parallel sucrose gradients which contained 50 mM Tris-HAc, 0.3 M KCl, 10 mM EDTA, pH 7.4. Lysates and column fractions were diluted with an equal volume of 0.5 M Tris-HAc, 0.6 M KCl, 0.2 0.2 M EDTA, pH 7.9. Precipitates were dissolved in a two-fold dilution of this same buffer.

RNA isolation

Column fractions containing ^{3}H-uridine-labeled RNA to be assayed for poly A content were pooled as indicated in Results, made 70% v/v ethanol, precipitated at -20°C overnight, and collected by centrifugation at 10,000 g for 10 min. Pellets were dried and resuspended in 1-2 ml of NETS buffer (0.1 M NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5% SDS, pH 7.4). Proteinase K (Merck) was added to a final concentration of 100 μg/ml and samples were incubated for 1 hr at 37°C. An equal volume of NETS-saturated phenol was added and samples were vortexed intermittently for 5 min. A second volume of NETS-saturated chloroform-isoamyl alcohol (24:1) was added, and the samples were vortexed intermittently for 5 min. The phases were separated by centrifugation at 3,000 g for 5 min. The aqueous phase and interphase were re-extracted two times with chloroform-isoamyl alcohol. The RNA was precipitated from 70% ethanol, 0.1 M NaCl, and reprecipitated 2-3 times.

Measurement of poly A content by dot blot hybridization

Poly A content of RNA fractions was assayed by RNA dot blot hybridization using nitrocellulose filters as described by Thomas (5). RNAs at a concentration of 1-2 mg/ml in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, were boiled for 4 min, quick-cooled on ice, and spotted in volumes of 2 μl onto dry nitrocellulose filters previously saturated with 20x SSC. Poly A_{30} (Collaborative Research), poly U (Miles), and E. coli tRNA and rRNA (Sigma) were included as controls for each hybridization. After spotting, the filters were air-dried and baked for 6-12 hr at 75°C.

For use as a probe, poly U was synthesized and sheared to ~40 NT as described by Angerer and Angerer (6), and 5' termini were labeled with [γ-^{32}P]-ATP (2,000 Ci/m mole; Amersham) using polynucleotide kinase (BRL) as described by Spradling (7). The specific activity of poly U was adjusted to 2 x 10^5 cpm/μg with unlabeled poly U (Miles).

Before hybridization, dot blots were washed twice with 100 ml of 0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4. Hybridization was carried out in sealed plastic bags in a volume of 80-100 μl/cm^2 of 0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 6 mM SDS, 6 mM Na_4PP_i, 2 mM EDTA, 0.1% Ficoll 400, 0.1% bovine serum albumin, 0.1% polyvinyl pyrollidone, pH 7.4. Poly U probe was heated to 85°C in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, for 3 min and added at an estimated 10-fold mass excess over the amount of poly A on the filter. Hybridiation was at 33.5°C for 16-18 hr. After hybridization, filters were washed 4 times with 0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 0.01% SDS, pH 7.4, for 30 min each at 33.5°. Filters were then air-dried and autoradiographed at

-70°C with Kodak Royal Xomat XRP-5 film and one Dupont Cronex Lighting Plus intensifying screen.

Autoradiograms were quantitated with an LKB soft laser densitometer using a slit width 80-90% of the diameter of the average spot. To verify that the hybridization signal was in the linear range of film sensitivity, a control hybridization with known amounts of poly A (usually 5 spots containing 0.25 to 4 ng) was included in each experiment. After the autoradiogram was quantitated, the amount of ^{3}H-labeled RNA in each spot was determined as follows. The filters were washed with water to remove salt, air-dried, and the spots excised and counted in Spectrafluor (Amersham) in triton-toluene (1:2).

RESULTS

Characterization of polysomes in exponentially growing and starved cells

In logarithmically growing cells harvested at 1-1.5 x 10^5 cells/ml, approximately 96% of ribosomes are in polysomes (Fig. 1A). The average polysome size determined from the mass distribution of ribosomes is approximately 10 (Table 1, line a). Thus, log-phase cells provide a source of nearly pure polysomes for developing isolation procedures which optimize recovery and minimize alteration of the polysome profile. In starved cells the rate of protein synthesis is 30 to 50-fold lower than in log-phase cells (F. Calzone, manuscript in preparation) and, in contrast to log-phase cells, only about 4% of ribosomes are in polysomes. Thus, starved cells provide a sensitive system for assaying the purification of polysomes from cells in which the majority of ribosomes are monosomes.

The level of polysomes in starved cells is too low to allow an accurate estimate of the average polysome size from the mass ditribution of ribosomes (Fig. 1B) because the dominant monosome peak obscures the polysome region of the gradient. We determined the average polysome size by pulse labeling nascent polypeptides with radioactive amino acids (Fig. 1C). The specificity of this label is indicated by the fact that at least 91% of the nascent label which sediments in the polysome region was released to the top of the gradient by pretreatment with EDTA (Fig. 1I); similar results were obtained with puromycin release (data not shown). The average polysome size for starved cells is 6-8.

These measurements of the fraction of ribosomes in polysomes and the average polysome size obtained for crude cell homogenates (10,000 g sup.) provided a reference for the development of methods for purification of

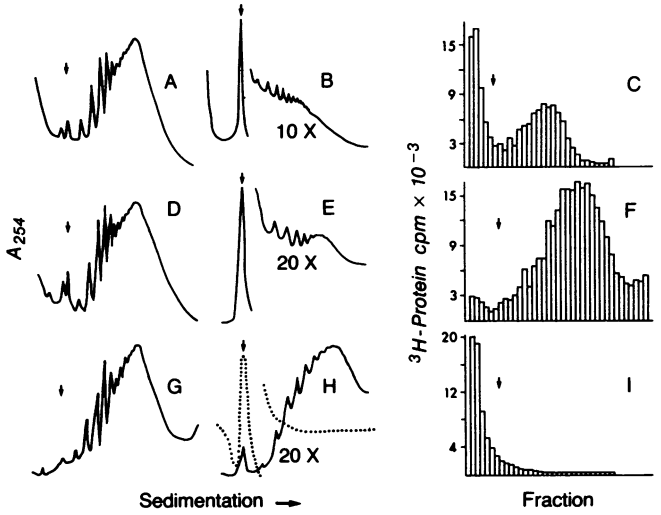

Figure 1. Sucrose gradient analyses of polysome fractions: A) log-phase cell, 10,000 g supernatant; B) starved cell, 10,000 g supernatant; C) starved cell, 10,000 g supernatant, ^{3}H-amino acid nascent label; D) log-phase cell, Bio-Gel A 1.5 m excluded volume; E) starved cell, Bio-Gel A 1.5 m excluded volume; F) starved cell Bio-Gel A 1.5 m excluded volume; H) starved cell, Bio-Gel A 15 m (solid line, excluded; dotted line included volume); I) starved cell, 10,000 g supernatant ^{3}H-nascent label EDTA treated. The break in the curves in B, E, and H (dotted line) indicates a change in scale. The arrows indicate the position of monosomes in each gradient profile.

polysomes which were unaltered by degradation or selection for large polysomes.

Chromatographic purification of total ribosomes

The elution profile from Bio-Gel A 1.5 m (monitored optically by A_{254} or isotopically by ^{3}H-RNA) was essentially the same for log-phase and starved cells, despite the large difference in the fraction of ribosomes on polysomes (Fig. 2A and B). The fraction of labeled RNA recovered in the excluded peak was 87% and 75% for log-phase and starved cells, respectively. Estimates of the fraction of total cellular RNA which is ribosomal (log-phase 92%, starved 87%; (8)) indicate recoveries of 95% and 86% for log-phase and starved cells, respectively. Analysis on sucrose gradients (Fig. 1D and E) showed that the average polysome size and the percentage of ribosomes in polysomes for the excluded fractions were indistinguishable from those of unfractionated preparations (Table 1, lines b and e).

Chromatography on Bio-Gel A 1.5 m provides a significant purification of

Table 1: Properties of Polysome Fractions

Cells	Fraction	Percent cytoplasmic RNA in fraction[1]	Percent cytoplasmic protein in fraction[1]	$\frac{^{14}C\text{-protein}}{^{3}H\text{-RNA}}$ relative to pure ribosomes[2]	Average polysome size[3]	Percent ribosomes in polysomes[3]
a. Log-phase	10,000 g supernatant	100	100	4.0	10 - 11	96
b. Log-phase	A 1.5 m excluded	87	47	2.1	10 - 11	95
c. Log-phase	A 15 m excluded	74	23	1.2	10 - 11	99
d. Starved	10,000 g supernatant	100	100	3.6	6 - 8	4 - 5
e. Starved	A 1.5 m excluded	75	41	1.9	6 - 8	4 - 5
f. Starved	A 15 m excluded	6	10	6.0	7 - 8	97
g. Starved	A 15 m excluded, pH 5.3 precipitated			1.8		

[1]Percents of cytoplasmic RNA (^{3}H-uridine) and cytoplasmic protein (^{14}C-leucine) were determined by monitoring recovery of radioactivity in each fraction.

[2]The ratio of ^{14}C-protein/^{3}H-RNA for each fraction was divided by the same ratio for ribosomal subunits purified by EDTA release and column chromatography.

[3]Average polysome size and fraction of ribosomes in polysomes were determined from polysome profiles shown in Figure 1 as described in Materials and Methods.

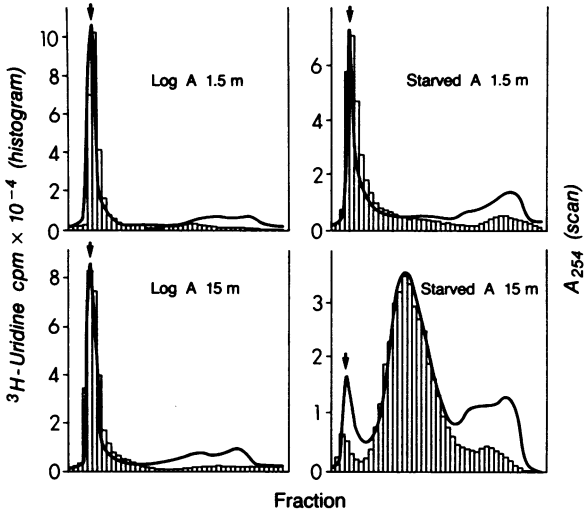

Figure 2. Elution profiles of log-phase and starved cell extracts fractionated on Bio-Gel A 1.5 m and A 15 m. Solid lines represent A_{254}; histograms indicate cpm in RNA labeled continuously with ^3H-uridine. The arrow indicates the peak of the excluded fraction.

ribosomes from soluble protein. To estimate this purification cells were labeled continuously during growth with ^{14}C-leucine and ^3H-uridine. Under the conditions used, greater than 95% of each isotope in the lysate is TCA precipitable. Comparisons of the ratio of $^{14}C/^3H$ in the excluded fraction to the same ratio for the original lysate and for highly purified ribosomal subunits indicate that about 50% of the excluded protein is ribosomal and that this chromatographic step removes 50-60% of the contaminating soluble protein.

Analysis of the size distribution of active ribosomes by pulse-labeling nascent peptides is greatly facilitated by prior purification on Bio-Gel A 1.5 m. For example, the radioactively labeled material which obscured the analysis of nascent label in monosomes and small polysomes in starved cells (Fig. 1C) is almost eliminated after one fractionation on Bio-Gel A 1.5 m (Fig. 1F).

The concentration of ribosomes was reduced less than two-fold by chromatography on Bio-Gel A 1.5 m. Therefore, no concentration step was necessary before subsequent analysis on sucrose gradients. Ribonuclease activities present in the initial homogenate are largely removed from the excluded fraction since these polysomes can be incubated up to 48 hours on ice

in column buffer without significant degradation (data not shown).

Chromatographic purification of polysomes

Bio-Gel A 15 m efficiently separated polysomes from monosomes in addition to purifying polysomes from ribonuclease and other soluble proteins. Polysomes were excluded from Bio-Gel A 15 m as indicated by the elution profile of an extract from log-phase cells (Fig. 2C). In this case, 74% of the labeled RNA loaded was excluded from the column (Table 1, line c). Sucrose gradient analysis (Fig. 1G; Table 1, line c) indicated that the size distribution of polysomes in this fraction was indistinguishable from that determined for crude lysates of log-phase cells (Fig. 1A; Table 1, line a). Monosomes were partially included as is indicated by the elution profile for starved cell extracts shown in Figure 2D. Only 6% of the ^{3}H-RNA loaded was recovered in the excluded fractions, in agreement with the percentage of ribosomes on polysomes in these cells. Sucrose gradient analysis (Fig. 1H) demonstrated that the excluded fraction for starved cells was 97% polysomes and that the large partially included peak contained monosomes. Thus, even when the fraction of ribosomes in polysomes is less than 5%, chromatography on Bio-Gel A 15 m results in virtually complete separation of polysomes from monosomes. Polysomes purified from starved cells by this method had an average size essentially the same as those in the crude lysate. We conclude that chromatography on Bio-Gel A 15 m effectively purifies polysomes with no detectable loss of small polysomes.

The purification of polysomes from non-ribosomal material on Bio-Gel A 15 m was significantly better than that obtained with Bio-Gel A 1.5 m. Only 23% of the ^{14}C-labeled bulk protein from log-phase cells co-eluted with polysomes (Table 1, line c). Analysis of the $^{14}C/^{3}H$ ratios indicated that at least 85% of the protein in this fraction was polysomal. The excluded fraction also contained a glycogen-like material. In starved cells, where the fraction of ribosomes in polysomes is low, it contributed up to 50% of the A_{254}. Some of this material and most remaining non-polysomal protein were subsequently removed from polysomes by precipitation at pH 5.3 (Table 1, line g) as described below.

Polysomes purified on Bio-Gel A 15 m were stable when incubated on ice for 48-72 hr in a buffer containing only 0.1% spermidine as a ribonuclease inhibitor. In contrast, polysomes in a crude cell homogenate were almost completely degraded after 1-2 hr. Since the average polysome size was not detectably reduced during purification (Table 1), it is unlikely that polysomes were degraded during chromatography. To more rigorously assess the

possibility of degradation and to calculate the efficiency of recovery of polysomes, ^{3}H-uridine labeled polysomes were purified from log-phase cells (Fig. 1G) and mixed with a starved cell homogenate. After chromatography on Bio-Gel A 15 m, 84% of the labeled polysomes was recovered in the excluded fraction (Fig. 3). The remaining 16% of the labeled polysomes was found in the trailing portion of the excluded peak and was not degraded to monosomes. This experiment and the lack of an observable reduction in the average size of purified polysomes demonstrate the absence of significant degradation of polysomes during purification.

Precipitation of polysomes at pH 5.3

To facilitate chromatographic preparation of large amounts of polysomes, it was advantageous to have a rapid method for concentrating ribosomes from large volumes. Once polysomes have been purified from most soluble protein, they can be precipitated at pH 5.3, pelleted by centrifugation at 10,000 g for 10 min, and resuspended wthout degradation as shown for log-phase polysomes in Figure 4A (compare to Fig. 1G). The integrity of the polysomes is also demonstrated by the observation that 95% of the labeled nascent polypeptides remained associated with polysomes after precipitation and resuspension (Fig 4B).

Precipitation of polysomes at pH 5.3 required 10 to 15 min at 0°C and was

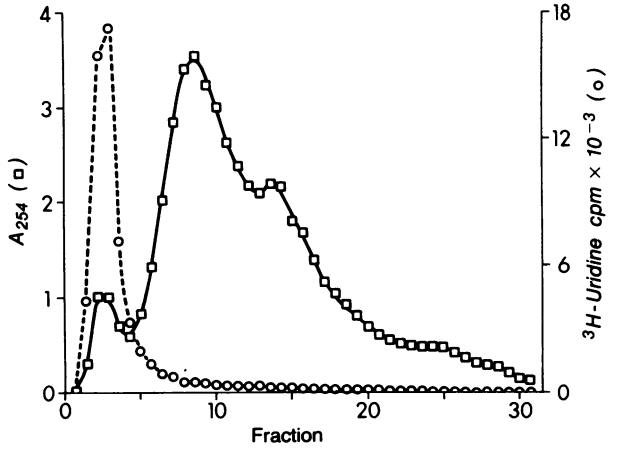

Figure 3. Test for degradation of polysomes during chromatography on Bio-Gel A 15 m. Polysomes were prepared from log-phase cells labeled continuously with ^{3}H-uridine mixed with a crude extract from unlabeled starved cells and rechromatographed on Bio-Gel A 15 m (o, log-phase ^{3}H-polysomes; □ , starved cell A_{254}).

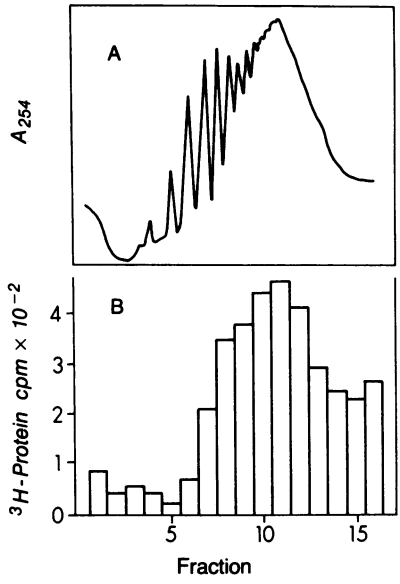

Figure 4. Sucrose gradient analysis of polysomes precipitated at pH 5.3. Polysomes were prepared from log-phase cells by chromatography on Bio-Gel A 15m, precipitated at pH 5.3, resuspended and analyzed on sucrose gradients. A) A_{254}; B) cpm in ^{3}H-nascent polypeptides.

completely efficient at concentrations of 2.5 A_{260} or greater. The efficiency of precipitation at lower concentrations can be increased by longer incubation time or by centrifugation at higher g force. For example, polysomes at a concentration of 0.8 A_{260} can be quantitatively recovered by centrifugation at 24,000 g for 10 min. The precipitation of ribosomes is dependent on $MgCl_2$ concentration. In contrast to chicken ribosomes (9, 10) the precipitation of Tetrahymena ribosomes at pH 5.3 is inhibited by high concentrations of $MgCl_2$, and the concentration dependence differs for polysomes and monosomes (Fig. 5). The maximum $MgCl_2$ concentration allowing complete precipitation of polysomes was about 60 mM, while that for monosomes was 20 mM. Similarly, the concentration of $MgCl_2$ required to resuspend polysomes and monosomes after precipitation was different. While polysomes can be resuspended in buffers containing as low as 5 mM $MgCl_2$, monosomes require at least 50-60 mM. The lower $MgCl_2$ maximum for precipitation of monosomes was labile to repeated cycles of precipitation.

The differential effect of $MgCl_2$ can be exploited to further purify polysomes from monosomes as demonstrated by the following experiment.

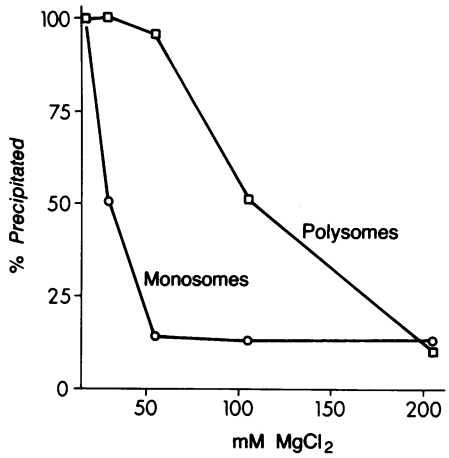

Figure 5. The effect of $MgCl_2$ concentration on the precipitation of polysomes and monosomes. Starved cell monosomes and log-phase polysomes were prepared by chromatography on Bio-Gel A 15 m, and the effect of $MgCl_2$ concentration on their precipitation determined as described in Methods.

Monosomes labeled with ^{3}H-uridine were purified from starved cells and mixed with increasing amounts of polysomes from log-phase cells. Precipitation was carried out in 50 mM $MgCl_2$ which allows precipitation of about 95% of the polysomes but only about 20% of the monosomes. At all ratios of polysomes to monosomes tested (0.154 to 1.37) precipitation of polysomes occurred without significant coprecipitation of monosomes (Table 2), indicating that at the concentration of total ribosomes tested, selective precipitation of polysomes is not affected by the ratio of monosomes to polysomes.

Purification of EDTA released mRNPs

Because many polysome preparations are contaminated with nuclear RNA (RNP), isolation of bona fide polysomal mRNA usually requires two sucrose gradient centrifugation steps (11). In the first step, polysomes are separated from smaller cytoplasmic and nuclear RNPs. mRNPs are then released from polysomes with EDTA and separated from more rapidly sedimenting contaminants on a second sucrose gradient. A similar strategy was adopted to purify polysomal mRNA by column chromatography (3). Polysomes from log-phase cells were precipitated, resuspended in 0.2 M KCl and 50 mM EDTA, and rechromatographed on Bio-Gel A 15 m. About 90% of the A_{254} in polysomes was shifted from the excluded to the partially included fraction. The behavior of mRNP during this treatment was studied by monitoring the elution of poly A^+

Table 2: Selective Precipitation of Polysomes at pH 5.3 from Mixtures of Polysomes and Monosomes

		Sample				
		1	2	3	4	5
a.	Concentration of ^{3}H-monosomes (A_{260})	2.11	2.14	2.31	2.12	2.22
b.	Concentration of polysomes (A_{260})	0.32	0.97	1.95	2.92	--
c.	Polysomes A_{260}/Monosomes A_{260}	0.154	0.455	0.844	1.37	--
d.	Percent monosomes precipitated (^{3}H)	17.7	20.3	24.8	18.9	15.0
e.	Percent polysome A_{260} precipitated†	95.0	102	101	96.3	--

†Corrected for the A_{260} contribution of monosomes which was determined from the ^{3}H cpm in each fraction and the specific activity of the monosome preparation.

RNA. Polysomes were purified (A 15 m) from log-phase cells labeled with ^{3}H-uridine (Fig. 6A). In this experiment, 71% of the labeled RNA loaded was recovered in the excluded volume. Column fractions were assayed for poly A content by RNA dot blot hybridization with a ^{32}P-poly U probe (Fig. 6B). About 63% of the poly A^+ RNA was excluded with polysomes. After EDTA treatment and rechromatography on Bio-Gel A 15 m, 93% of the RNA mass (^{3}H-uridine) and 90% of the polysomal poly A^+ RNA were shifted from the excluded to the partially included fraction (Fig. 6C and D), demonstrating that polysomal mRNPs behave as expected.

mRNA loading in starved Tetrahymena

As indicated in Figure 6 most of the cytoplasmic poly A^+ RNA in log-phase cells is loaded on polysomes. The relative amounts of polysomal and non-polysomal mRNA in starved cells were measured by the same method (Fig. 6E and F). Only 7% of the RNA mass and 14% of the poly A^+ RNA were found to co-purify with polysomes. Thus, in contrast to log-phase cells, most of the poly A^+ RNA in starved cells is not loaded on polysomes.

DISCUSSION

Column chromatographic methods for the purification of polysomes offer several advantages over traditional methods utilizing sucrose gradient centrifugation. Purification of polysomes by column chromatography requires

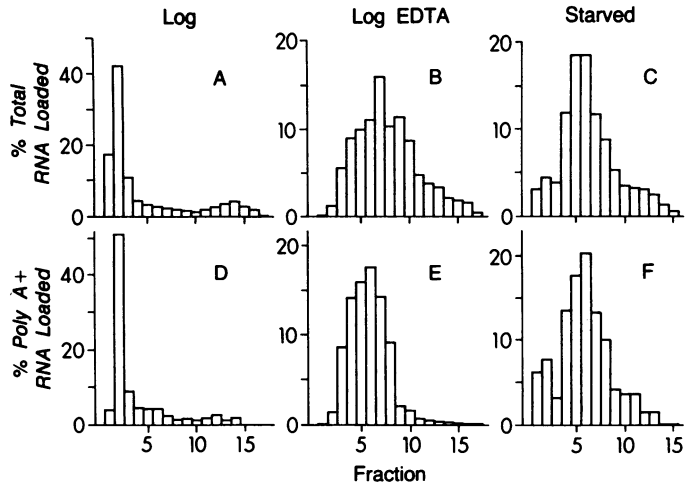

Figure 6. Loading of poly A^+ RNA and total ribosomes in log-phase and starved cells. Extracts from log phase (A and B) or starved (E and F) cells continuously labeled with ^{3}H-uridine were chromatographed on Bio-Gel A 15 m. Polysomal (excluded) fractions from log-phase cells were dissociated with EDTA as described in Materials and Methods to release mRNPs and rechromatographed (C and D). The distribution of total RNA (which is 90% ribosomal) is shown in A, C, and E. The distribution of poly A^+ RNA was determined from measurements of relative poly A content of the column fractions by dot blot hybridization with ^{32}P-poly U, and from the percent of the total RNA present in each fraction.

less work, is less expensive and can easily be applied to large quantities. Column buffers avoid the use of sucrose solutions which are undesirable for some subsequent manipulations.

The methods described here extend previous methods which used Sepharose-2B (2, 3). We have shown that Bio-Gel A 1.5 m can be used to purify total ribosomes from the majority of soluble protein without altering the percentage of ribosomes in polysomes or the polysome profile. Polysomes prepared on Bio-Gel A 15 m are free of monosomes and smaller mRNPs but are not selectively enriched for large polysomes. The 4% of ribosomes loaded on polysomes in starved Tetrahymena can be prepared essentially free of monosomes in a single chromatographic step.

These column chromatographic methods can also be applied to the preparation of EDTA or puromycin released mRNP. Such a procedure is frequently employed to remove nuclear contaminants from polysomal mRNA (11). The rapid synthesis and export of ribosomes in Tetrahymena (8) makes it

difficult to evaluate the extent of nuclear contamination in released mRNPs. However, RNA excess hybridization experiments (unpublished observation) have demonstrated that polysomal mRNA is sufficiently purified from nuclear RNA contamination for complexity measurements.

Once polysomes have been purified from most cytoplasmic protein, they can be precipitated at pH 5.3 without degradation. Previous work using chicken ribosomes emphasized the importance of high $MgCl_2$ (> 0.1 M) at either high or low pH for the precipitation of polysomes (9, 10). We have not been able to precipitate Tetrahymena polysomes in the presence of high $MgCl_2$ concentrations under any conditions. Interestingly, Tetrahymena monosomes and polysomes have maximum $MgCl_2$ concentrations for precipitation which differ by at least 2 to 3-fold. While the basis for this difference is unclear, we have noted that monosomes derived from polysomes by brief RNase A digestion behave like native monosomes.

We have made a precise estimate of the fraction of ribosomes in polysomes for starved Tetrahymena. While log-phase cells have about 95% of ribosomes loaded on polysomes, this is reduced to 3-5% in starved cells. This reduction is not due to a corresponding reduction in the number of mRNAs in starved cells. Poly A^+ RNA constitutes the same fraction (about 3%) of the cytoplasmic RNA in log-phase and starved cells (data not shown). Using the column methods described here, we have shown that while log-phase cells have most of the poly A^+ RNA (presumably mRNA) loaded on ribosomes, starved cells translate only a small fraction of the existing mRNA. Such a distribution of mRNA is characteristic of several eukaryotic systems where translational control mechanisms regulate the rate of protein synthesis by limiting the fraction of cytoplasmic mRNA available for translation (for example, see 12).

The large increases in the fraction of non-polysomal mRNA during starvation of Tetrahymena may be a simple response to nutrient deprivation. However, a period of starvation in 10 mM Tris is also a signal for Tetrahymena thermophila to initiate the sexual phase of its life cycle (13). It is possible that during this period, the cell stores (specific?) mRNAs that will be used later in development.

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REFERENCES

1. Davidson, E. H. (1976). Gene Activity in Early Development. 2nd Edition. Academic Press, Inc.
2. Tai, P. -C., Wallace, B. J., Herzog, E. L., and Davis, B. D. (1973). Biochemistry 12, 609-615.
3. Sniderski, R. E., Johnson, S. A., Larkins, B. A., and Graham, D. E. (1979). Nuc. Acids Res. 6, 3685-3701.
4. Oleinick, N. L., Rustad, R. C., and Kuncio, G. S. (1974). Biochim. Biophys. Acta 366, 215-223.
5. Thomas, P. S. (1980). Proc. Natl. Acad. Sci. USA 77, 5201-5205.
6. Angerer, L. and Angerer, R. (1981). Nuc. Acids Res. 9, 2819-2840.
7. Spradling, A. C., Digan, M. E., Mahowald, A. P., Scott, M., and Craig, E. (1980). Cell 19, 905-914.
8. Hallberg, R. L. and Bruns, P. J. (1976). J. Cell Biol. 71, 383-394.
9. Palmiter, R. D. (1974). Biochemistry 13, 3606-3615.
10. Bast, R. E., Garfield, S. A., Gehrke, L., and Lau, J. I. (1977). Proc. Natl. Acad. Sci. USA 74, 3133-3137.
11. Galau, G. A., Britten, R. J., and Davidson, E. H. (1974). Cell 2, 9-20.
12. Jenkins, N. A., Kaumeyer, J. F., Young, E. M., and Raff, R. A. (1977). Dev. Biol. 63, 279-298.
13. Bruns, P. J. and Brussard, T. B. (1974). J. Exp. Zool. 188, 337-344.